INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: METHOD FOR PREPARING, ISOLATING AND SEQUENCING POLYNUCLEOTIDES

(57) Abstract

The present invention provides a method for sequentially preparing, isolating and sequencing a polynucleotide acid strand comprising the steps of: (1) preparing a solution of a polynucleic acid target fragment having attached thereto a separating label, (2) mixing the solution with a support matrix having attached thereto a group cooperatively bindable with the separating label, (3) immobilising the polynucleic acid fragment on the support matrix via binding of the separating label and the group, (4) purifying the immobilised strand, and (5) treating the immobilised strand for sequencing, wherein the preparing step, the immobilising step and at least part of the treating step are conducted in the same vessel.

Method For Preparing, Isolating and Sequencing Polynucleotides

The present invention relates to a method of preparing, isolating and sequencing polynucleotides.

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The method is particularly useful for isolating and sequencing polynucleotide fragments prepared by a modified polymerase chain reaction (PCR) technique that is disclosed in International patent application PCT/GB91/00803.

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Since its introduction, the di-deoxy DNA sequencing method (Sanger et al [1977] PNAS 74 5463) has found wide spread acceptance and has even been successfully utilised for sequencing large viral genomes (Baer et al [1984] Nature 310 15 207). Numerous improvements have been made in an attempt to permit the elucidation of more than one DNA sequence from a single clone. Most of these improvements are related to improving the electrophoretic resolution and rapid computer assisted data entry. The advent of fluorescently labelled 20 sequencing reagents has enabled the combination of both of these improvements, thereby permitting automation of electrophoresis and gel reading. However, because these improvements are aimed at facilitating gel running and data entry, the limiting factor in analysing polynucleotide 25 fragments actually becomes the step of preparing the fragments for sequencing.

An improvement in target fragment preparation was reported by Kristensen (Kristensen et al [1987] NAR 15 5507). This 30 improvement involves the precipitation of M13 phage with acetic acid, followed by the recovery and subsequent disruption of the phage on glass fibre discs. The released DNA is then eluted with a low salt buffer, which requires neither phenol extraction nor ethanol precipitation. This 35 improvement is reported to have a potential for automation (Zimmerman et al [1989] Meth. Mol. Cell. Biol. 1 29) but suffers from the fact that it is limited to the use only of M13 phage and its derivatives.

Also, the improvement is further limited because it is
dependent upon bacterial growth (which usually has to occur
over a period of six hours) and a DNA purification step.

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The polymerase chain reaction (PCR) (Saiki, R. et al [1985]
Science 230 1350 and [1987] Science 239 487) offers one way
of circumventing the need for having a bacterial culture for
preparing amplified amounts of a target fragment for
10 sequencing.   However, there are some inherent problems
associated with the PCR technique.   For example, the
products of the PCR are often relatively short linear
molecules, which are usually less than 2kb in length. Also,
the products cannot be directly sequenced because of the
15 presence of residual dNTPs and primers in the reaction
mixture.   Also, there is a tendency for the separated DNA
strands to reanneal very quickly after alkali or heat
denaturation. However, reannealing can be hindered by using
10% DMSO (Winship [1989] NAR 17 1266).

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Other methods to overcome the annealing problem include the
complete removal of one strand or conducting asymmetric PCR
to produce an excess of one strand (Gyllensten et al [1988]
PNAS 85 7652).   Because of the unexplained template and
25 primer variability encountered in the Gyllensten method, an
alternative method of single strand template preparation
has been suggested (Higuchi et al [1989] NAR 17 5865).

The method of Higuchi depends on the phosphorylation of one
30 of the PCR primers which then targets the strand to be
digested, following PCR, by λ exonuclease III.   Other
workers have used ^{32}P end-labelled PCR or internal primers
prior to the sequencing step (Wrischnik et al [1987] NAR 15
529, Wong et al [1987] Nature 330 384).   Also, direct
35 sequencing from low-melt agarose has been conducted (Kretz
et al [1989] NAR 17 5864).

Accordingly, current techniques for the preparation, isolation and sequencing of nucleic acid fragments include a first step of amplifying a target nucleic acid fragment (i.e. the fragment of interest), for example, by use of the
5   PCR technique (Saiki, R. *et al* [1985] Science 230 1350 and [1987] Science 239 487). Next, the copies of the target fragment produced by PCR amplification are isolated, for example, by gel electrophoresis. The isolated strands are then sequenced by use of standard di-deoxy labelling or
10 radiolabelling techniques or standard chemical degradation techniques.

However, it is generally known and accepted that it is not practicable to conduct each of the present steps in a
15 simple, reliable and straightforward run from start (i.e. preparing amplified amounts of the target fragment) to finish (i.e. sequencing the prepared target fragment). It is also accepted that it is not possible to have one machine that could carry out each of the above steps in a continuous
20 run. This is because the amplification, isolation and sequencing steps are three quite separate and incompatible steps that have to be conducted in different vessels. Also, these steps have to be performed using quite different volumes of samples and concentration levels. Also, some of
25 these methods require time consuming additional steps prior to sequencing and, often, the results from the sequencing analysis can vary according to the protocol used.

The major drawbacks that prevent such a reliable and
30 continuous method from being devised therefore stem from the inherent problems associated with each of the present steps. For example, the amplification step often requires extremely dilute samples of the target fragments, whereas the isolation step thrives on concentrated samples. Also, each
35 of the above steps using current methods employs solutions having volumes of approximately 0.01 ml and upwards, with the amplification step requiring the largest volumes.

Thus an automated machine using current techniques would
have to handle large volumes.   This is clearly
disadvantageous because the machines themselves would be
large and costly to produce, maintain and run.

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It is therefore apparent that none of the forementioned
methods is amenable to large-scale sequencing of genomic
products such as those involved in Human Genome Project.

10 It would be extremely advantageous, therefore, to have a
method of, and also a machine for, preparing, isolating and
sequencing a target fragment.   It would be even further
advantageous to have such a method wherein each of those
steps could be conducted at the μl level.   In order to
15 achieve this goal, the present method builds upon and
refines a method devised by Hultman et al ([1989] NAR 17,
4937-4946).

The Hultman method was devised for the sequencing of
20 polynucleotide fragments.   The method yields a purified
polynucleotide fragment attached to a solid support, which
can then be readily sequenced by standard Sequenase (trade
mark) or Taquenase (trade mark) methodology.

25 In brief, the Hultman method uses streptavidin coated
magnetic beads to immobilise biotin labelled polynucleotide
fragments.   Once the fragments are immobilised, any unwanted
products and artifacts can be easily removed by washing the
beads.   The fragments can thus be isolated and purified by
30 a straightforward and simple procedure.

In brief, the Hultman method of immobilisation includes the
following 6 steps:

35   1.   preparing magnetic beads containing covalently
                    coupled streptavidin (e.g. Dynabeads M280-
                    Streptavidin, which can be obtained from Dynal
                    AS, Norway),

2. preparing a target double stranded (ds) DNA fragment (e.g. from a plasmid or a sample of genomic DNA),

5 3. binding a biotin label to either the 5' end or the 3' end of one of the strands of the ds DNA fragment (see below),

4. mixing the prepared beads and the biotin
10 labelled ds fragment under conditions to allow the fragment to bind to the beads via the biotin label (i.e. immobilising the labelled fragments),

15 5. isolating the complexed bead-fragments by use of a magnet (e.g. neodymium-iron-boron permanent magnet), and

6. optionally washing the isolated complexed
20 bead-fragments.

The binding of the biotin label (step 3) can be performed by either a filling-in of nucleotide overhangs of a sticky-ended fragment with biotin-dUTP and polymerase (i.e.
25 extension of the target strand) or by using a biotinylated primer that anneals to a universal cassette that is ligated to the target strand during an *in vivo* amplification operation (e.g. during an exponential PCR amplification).

30 The fragments prepared by the alternative biotin binding methods have different orientations when immobilised on the beads.

In this regard, the filling-in step using biotin-dUTP
35 produces a biotin label at the 3' end of a strand of the target fragment. Therefore, when the target strand is immobilised on the beads, the 5' end of this target strand is distanced away from the beads.

In contrast, the use of a biotin labelled primer that anneals to a ligated universal cassette produces a biotin label at the 5' end of a template strand of the target

5   fragment.   Therefore, when this target strand is immobilised, the 3' end of the target strand is distanced away from the beads.

The fragments to which the biotin labels bind can be

10   prepared, for example, by PCR exponential amplification. After PCR amplification, the biotin labelled strands of the fragments can be bound to the beads. The PCR products can be easily purified because the non-biotinylated strands of the fragments can be selectively removed by alkaline

15   treatment. The purified polynucleotide fragment strand can then be sequenced.

In the Hultman method, the immobilised biotinylated polynucleic acid fragment strand is sequenced by a procedure

20   including the following 12 steps:

1.   denaturing the immobilised double stranded (ds) fragment to a single stranded (ss) polynucleic acid fragment with 0.15 M NaOH for

25   5 minutes,

2.   washing the beads with 0.15 M NaOH and H_2O,

3.   mixing the washed immobilised ss fragment with

30   2 pmol of a labelled (i.e. ^{32}P labelled or fluorescently labelled) sequencing primer in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and 0.1 mg/ml BSA in a total volume of 0.01 ml,

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4.   heating the annealed mixture to 65°C and allowing it to cool to room temperature,

5.   adding 0.001 ml DTT/NaCl (0.1 M DTT/0.8 M
      NaCl) and 4 units of T7 DNA polymerase, and
      then adjusting the volume to 0.015 ml,

5   6.   mixing 0.0035 ml of the mixture from step 5
            with 0.0025 ml of a respective nucleotide
            mixture (see below),

      7.   incubating the reaction mixture for 10 minutes
10   at 37°C,

      8.   removing the supernatant after extension has
            been completed and washing the beads in water,

15   9.   adding 0.003 ml of a formamide/sequencing dye
            mixture (deionised formamide consisting of 10
            mM EDTA, pH 7.5 0.3% (w/v) xylen cyanol ff and
            0.3% (w/v) Bromophenol Blue) and incubating
            at 37°C for 15 minutes,

20
      10.   removing the supernatant mixture (which
            includes the newly synthesised oligo-
            nucleotides) and diluting the mixture with
            0.003 ml water,

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      11.   loading 0.002 ml of the diluted supernatant
            mixture on a 4% standard electrophoresis
            polyacrylamide gel or on a 7 % sequencing gel,
            which is then run using an automatic
30   sequencing apparatus, and

      12.   detecting the bands after electrophoresis.

35 The typical nucleotide concentrations and mixtures used in
   step 6 are 0.080 mM of each dNTP, 0.0063 mM of the
   respective ddNTP, 50 mM NaCl and 40 mM Tris-HCl (pH 7.5).

Despite the apparent simplicity of the Hultman method, there
are a number of associated inherent problems with the
immobilising and sequencing steps.   In particular, the
method requires large quantities of Taq polymerase,
5   biotinylated primer and streptavidin beads.   Also, the
sequencing stage requires a labelled primer and a purified
template.

We have considerably modified the Hultman method to overcome
10   its inherent problems.   In particular, our method is
conducted on the micro volume scale thereby permitting a
reduction in the amount of Taq enzyme, primer and
streptavidin beads required.

15   Our method also allows the direct addition of λ phage, m13
phage or bacterial cells to the PCR reaction without any
additional purification of the PCR template.   Thus it is
possible to circumvent the need for bacterial growth and DNA
template preparation.   This makes the whole process very
20   amenable to automation.

Indeed, using the present method, it is possible to carry
out a PCR amplification reaction or a number of the PCR
amplification reactions in a specially designed microtitre
25   plate PCR machine and to isolate the products therefrom and
to sequence the products without having to transfer the
products to different reaction vessels.

According to the present invention, we provide a method of
30   sequentially preparing, isolating and sequencing a
polynucleic acid strand comprising the steps of:

1.   preparing a solution of a polynucleic acid target
     fragment having attached thereto a separating label,

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2.   mixing the solution with a support matrix having
     attached thereto a group cooperatively bindable with the
     separating label,

3. immobilising the polynucleic acid fragment on the
    support matrix via binding of the separating label and
    the group,

4. purifying the immobilised strand, and

5. treating the immobilised strand for sequencing,

    wherein the preparing step, the immobilising step and
    the (or at least part of the) treating step are
    conducted in the same vessel.

Preferably, all of the sequencing reaction is conducted in
the same vessel and the product is then sequenced on an
automated machine, such as Du Pont's Genesis 2000 machine.
This can be achieved by use of fluorescent dideoxy chain
terminators. The products of the treating step need only
then be run on one gel in order to establish the sequential
order of the nucleotides in the immobilised strand.

It is appreciated that if non-labelled dideoxy terminators
are used in the treating step in conjunction with $\alpha^{35}S$
labelled dideoxy terminators, the immobilised strands could
then be subjected to the respective radiolabelled
terminators in different vessels and at different times.

Preferably, the polynucleic acid target fragment is prepared
by a PCR amplification step, Advantageously, the PCR
amplification is conducted in a volume of between 5μl and
20μl, preferably 10μl.

Advantageously, one of the primers used in the amplification
step has attached thereto the separating label. Preferably,
the separating label is attached to the 5' end of the
primer. Conveniently, the primer with the attached label is
a primer that anneals to a specific region on the target
fragment. This ensures that only the target fragment is

isolated.

Preferably, the label is biotin and the support matrix is a
magnetic bead having streptavidin covalently coupled
5   thereto.   The streptavidin beads are particularly
advantageously because harsh conditions (e.g. strong
alkaline solutions) can be used to clean the immobilised
fragments without the immobilised fragments becoming unbound
from the beads.
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However, it is to be understood that any other appropriate
separating label and support matrix would suffice, for
example DNA binding proteins.

15 Preferably, the beads are added in the form of an aqueous
dispersion to the amplification mixture. A typical aqueous
dispersion has a volume of 15μl.

Advantageously, if the labelled target fragment is a double
20 stranded fragment, it can be denatured while it is bound to
the support matrix. However, it is clear that the target
fragment can be denatured prior to the addition of the
support matrix.

25 In both instances, only the labelled target fragments will
bind to the support matrix. A typical denaturation solution
is 20μl of 0.15M NaOH. The fragments bound to the beads can
then be washed with, approximately, 20μl solutions of 0.15M
NaOH and 40μl solutions of H_2O.
30

Advantageously, the purified fragments attached to the beads
are dispersed in approximately 5-10μl H_2O (preferably
7.5μl) and sequenced using approximately 5-12 μl of
sequencing solution (preferably 7-10μl).   A typical
35 sequencing solution comprises approximately 0.5μl of 10μM
forward primer, 2μl of 5x Sequenase buffer, 1μl of 100mM
DTT, 2μl of labelling mix (radiolabelled dideoxy terminators
or fluorescent dideoxy terminators), 0.5μl of ^{35}S dATP and

2μl of Sequenase; 4μl of which is mixed with 2.5μl of termination labelling mix.

The present method is suitable for a simple kit so that the
5 method can be conducted on a laboratory workbench. The present method can also be conducted by an automated machine, particularly as the amounts of reagents required are low. Also, such an automated machine could be supplied in kit form.
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The present method has a considerable number of advantages over existing methods. It is quicker than conventional methods, it yields better quality sequences using either ^{35}S and autoradiography or fluorescent di-deoxy chain
15 terminators (e.g. with the Du Pont Genesis 2000™ sequencing machine) and it allows the rapid sequencing of any nucleic acid fragment, for example genomic DNA that can be, but need not be, cloned in a vector.

20 Also, the present method makes it possible to prepare the template DNA, perform the sequencing reactions and run a sequencing gel in one working day. In addition, the data obtained using the present method are of higher quality than those obtained from either conventional double or single
25 stranded DNA sequencing.

Moreover, the isolated target fragment template produced is extremely clean and therefore the sequence produced is also very clean. This is true both of ^{35}S-labelled DNA and
30 fluorescently labelled fragments.

A method according to the present invention will now be described by way of example only.

35

Step 1. Preparation by PCR amplification

The target fragment to be sequenced (which may be contained
within λ phage, M13 phage, yeast or bacterial cells) is
5   added to the 10μl reaction mixture, which comprises 1μl of
10x Cetus buffer, 0.5μl of 10μM biotinylated reverse primer,
1μl of 2mM dNTPs and 0.04 units of Taq polymerase. H_2O is
then added so that the total volume is 10μl.

10   Next the mixture is overlayed with 10-20μl light paraffin
oil, then heated at 95°C for 0.3 min, 55°C for 0.5 min and
72°C for 1 min for a total of 35 cycles, followed by heating
at 72°C for 3 min.

15   ## Step 2. Isolation of labelled target fragment

15μl of streptavidin coated magnetic beads in TE/NaCl are
added to the whole of the PCR reaction (including paraffin).
After mixing, the mixture is incubated at room temperature
20   for 15 min.

Then, optionally, the beads are washed four times with
TE/NaCl.

25   ## Step 3. Denaturation

The beads are incubated at room temperature for 5 min with
20μl of 0.15M NaOH. The beads are then washed with 20μl of
0.15M NaOH (if desired, the paraffin can be wholly or
30   partially removed at this stage) and then three times with
40μl of H_2O.

Step 4. Sequencing

35   The beads are resuspended in a total volume of 7.5μl of H_2O,
to which suspension is added 0.5μl of 10μM forward primer
and 2μl of 5x Sequenase buffer. The mixture is incubated at
65°C for 2 min, then ice cooled for 1 min. Next, 1μl of

100mM DTT, 2μl of diluted labelling mix (1:5 for longer
sequences, 1:20 for shorter ones), 0.5μl of ^{35}SdATP (0.5μCi)
and 2μl of diluted Sequenase (1:8) are added.

5 The mixture is then incubated at room temperature for 5 min.
4μl is transferred to the termination mixes (2.5μl). The
mixture is incubated at 37°C for 5 min and then washed with
20μl TE. Afterwards, the supernatant is removed and 4μl of
formamide dye mix is added. Following incubation at 80°C
10 for 5 min, the mixture is spun and the supernatant removed.
A sample (e.g. 3-4μl) of the supernatant is then loaded on
a buffer gradient sequencing gel.

The present method can be easily conducted in the
15 laboratory. In a typical example, a 96-prong replica-plated
comb transfers bacterial cells into the wells of a
thermostable polycarbonate microtiter plate. A programmed
robotic workstation dispenses 10μl of PCR mixture (200μM
dNTP's, 1.5mM MgCl$_2$, 50mM Tris-HCl pH 8.3, 0.01% gelatin,
20 1μM each of M13 Universal forward and reverse sequencing
primers, 0.8 Units Taq polymerase USB™) into the microtiter
wells and, following plating, overlays the solution with
40μl light mineral oil.

25 Next, the plate is incubated on a modified programmable
thermocycler adapted to accommodate such plates. The target
DNA is then exposed by heating the solution to 95°C for 2
minutes prior to 30 cycles of amplification (95°C - 0.5 min,
55°C - 0.5 min, 72°C - 1 min).

30
This present method allows the presence and size of inserts
in 96 clones to be determined within 4 hours.

We have also extended this protocol to the direct sequencing
35 of PCR products in which the amplification involves one
biotinylated primer. This product is bound to magnetic
streptavidin beads and washed with NaOH and H$_2$O. The
single-stranded fragment templates can be routinely

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sequenced either manually, using Sequenase™, or in a single
well with fluorescently labelled dideoxynucleotides, prior
to loading on a Du Pont Genesis 2000 automated DNA
sequencing machine.

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It is clear from the above, that the present method includes
the small-scale amplification of DNA template in which one
of the PCR primers has a biotin group at its 5' prime end.
This allows a single strand of DNA to be purified by binding
10 it to streptavidin-coated magnetic beads and washing with
alcohol. Because the reactions are carried out on a small
scale, the amount of Taq polymerase enzyme required is kept
to a minimum, thus reducing cost.

15 It is clear that the rapid automated screening and
sequencing strategies described here will contribute to both
small and large scale sequencing projects.

It will of course be understood that the present invention
20 has been described above by way of example only and that
modifications and variations can be made by the skilled
person without departing from the scope of the invention.

15

CLAIMS

1.   A method for sequentially preparing, isolating
and sequencing a polynucleotide acid strand comprising the
5   steps of:

(1) preparing a solution of a polynucleic acid target
    fragment having attached thereto a separating label,

10 (2) mixing the solution with a support matrix having
        attached thereto a group cooperatively bindable with the
        separating label,

(3) immobilising the polynucleic acid fragment on the
15   support matrix via binding of the separating label and
        the group,

(4) purifying the immobilised strand, and

20 (5) treating the immobilised strand for sequencing,

        wherein the preparing step, the immobilising step and
        at least part of the treating step are conducted in the
        same vessel.
25
2.   A method according to claim 1, wherein the
entire treating step is conducted in a single vessel and
sequencing is performed using an automated sequencing
machine.
30
3.   A method according to claim 1 or claim 2,
wherein the polynucleic acid target fragment is prepared by
a PCR amplification step.

35 4.   A method according to claim 3, wherein the
target fragment is prepared by performing a PCR
amplification directly on M13, λ phage or bacterial cells.

5.   A method according to claim 3 or claim 4,
wherein the separating label is comprised in a primer used
in the PCR amplification step.

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6.   A method according to any preceding claim
wherein the separating label is biotin and the support
matrix comprises a magnetic bead covalently. coupled to
streptavidin or avidin.

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7.   A method according to any preceding claim
wherein step 4 comprises denaturing the target fragment in
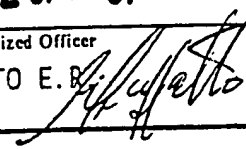an alkali solution to purify the labelled strand.

15 8.   A method according to any preceding claim
wherein sequencing is carried out in a volume of less than
$22\mu l$.

9.   A method according to any preceding claim
20 which is conducted on a microlitre volume scale.

10.   A method according to any preceding claim
which is conducted substantially entirely by an automated
machine.

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I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCLEIC ACIDS RESEARCH. vol. 17, no. 22, 25 November 1989, ARLINGTON, VIRGINIA US page 9498; J.P. SCHOFIELD ET AL.: 'A rapid semi-automated microtiter plate method for analysis and sequencing by PCR from bacterial stocks' see the whole document	1-10

A	EP,A,0 371 437 (ORION-YHTYMA OY) 6 June 1990 see column 5, line 15 - column 15, line 25	1-10

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 NOVEMBER 1991	2 6. 11. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LUZZATTO E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	NUCLEIC ACIDS RESEARCH. vol. 17, no. 13, 1989, ARLINGTON, VIRGINIA US pages 4938 - 4946; T. HULTMAN ET AL.: 'Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support' cited in the application see the whole document ---	1
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A	CLINICAL CHEMISTRY. vol. 35, no. 11, November 1989, WINSTON US pages 2196 - 2201; L.J.MCBRIDE ET AL.: 'Automated DNA sequencing methods involving Polymerase Chain Reaction' see the whole document esp. abstract ---	1
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		CA-A-	2004056	29-05-90
		JP-A-	2219600	03-09-90